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David P. Marancik [a] , Timothy J. Welch [a] , Timothy D. Leeds [a] & Gregory D. Wiens [a]
[a] National Center for Cool and Cold Water Aquaculture, 11861 Leetown Road , Kearneysville , West Virginia , 25430 , USA
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ARTICLE

Acute Mortality, Bacterial Load, and Pathology of Select Lines of Adult Rainbow Trout Challenged with *Weissella* sp. NC36

David P. Marancik, Timothy J. Welch,* Timothy D. Leeds, and Gregory D. Wiens*
National Center for Cool and Cold Water Aquaculture, 11861 Leetown Road, Kearneysville, West Virginia 25430, USA

Abstract

A challenge for improving disease resistance in fish through genetics is to understand specificity of resistance and whether selection for one pathogen alters the response to unrelated pathogenic microorganisms. Adult Rainbow Trout *Oncorhynchus mykiss* that had been bred for differential susceptibility to *Flavobacterium psychrophilum*, the causative agent of bacterial cold water disease (BCWD) and designated ARS-Fp-R (resistant), ARS-Fp-S (susceptible), and ARS-Fp-C (control line), as well as a pool of commercial-stock Rainbow Trout, were intraperitoneally challenged with *Weissella* sp. NC36. Clinical signs, survival, and innate mechanisms affecting disease resistance were monitored over 9 d. Acute disease signs included exophthalmia associated with retrobulbar inflammation and hemorrhage, cerebral hemorrhage, and mild to moderate granulomatous pericarditis. The ARS-Fp-R line did not demonstrate significant survival differences over a 9-d period compared with the ARS-Fp-C and ARS-Fp-S lines ($P \geq 0.09$) indicating that during the acute phase of disease, the resistance factors that limit BCWD do not confer cross protection against *Weissella* sp. NC36. The linear effect of body weight at challenge was statistically significant, as each 10-g increase in body weight increased the hazard of death by 1% ($P = 0.02$). Bacterial loads on day 3, assessed by splenic and cerebral CFU counts, did not differ between ARS-Fp-R and ARS-Fp-S trout and there was no correlation between CFU counts and body weight. These findings help elucidate the specificity of disease resistance in selectively bred lines and contribute to our understanding of disease caused by *Weissella* sp., a recently described pathogen found in cultured Rainbow Trout.

Enhancing disease resistance through selective breeding is an increasingly employed strategy to reduce fish morbidity and mortality in aquaculture (Ødegård et al. 2011; Gjedrem et al. 2012). A challenge of selective breeding is to understand specificity of disease resistance and whether selection alters the response to unrelated pathogenic microorganisms.

We have previously described a selective breeding program, initiated in 2005, to increase survival of Rainbow Trout *Oncorhynchus mykiss* following challenge with *Flavobacterium psychrophilum*, the causative agent of bacterial cold water disease (BCWD). Relative survival to intraperitoneal challenge with *F. psychrophilum* was moderately heritable and not adversely correlated with growth (Silverstein et al. 2009). The top performing families were subsequently chosen to produce a disease-resistant line of Rainbow Trout, designated ARS-Fp-R (Leeds et al. 2010; Wiens et al. 2013). Additionally, highly susceptible (designated ARS-Fp-S) and randomly bred control lines (ARS-Fp-C) have been used as a reference for phenotypic comparison (Wiens et al. 2013). In a small-scale, field-trial evaluation, the ARS-Fp-R line showed increased survival compared with ARS-Fp-C, ARS-Fp-S, and the commercial stocks during natural outbreaks of *F. psychrophilum* on fish farms (Wiens et al. 2013; G. D. Wiens, unpublished data).

As part of a broader effort to understand specificity of disease resistance in Rainbow Trout and to anticipate performance of R-line fish that will be used for commercial production, innate

*Corresponding authors: tim.welch@ars.usda.gov; greg.wiens@ars.usda.gov
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resistance to experimental challenge with *Weissella* sp. NC36 was examined. This pathogen is significant as it was isolated from commercially farmed Rainbow Trout during a severe disease outbreak in western North Carolina in 2011 (Welch and Good 2013). Disease was observed in 250- to 1,000-g fish with clinical signs of lethargy, skin darkening, exophthalmia, cerebral hemorrhage, and high mortality sustained over a period of 2 months.

Recent occurrences of Weissellosis in Brazil (Figueiredo et al. 2012) and China (Liu et al. 2009) are the first where *Weissella* sp. as a fish pathogen has been described and suggest Weissellosis represents an emerging disease in Rainbow Trout aquaculture (Welch and Good 2013). Experimental induction of disease via immersion (Figuiredo et al. 2009; Welch and Good 2013) and by cohabitation of healthy and diseased fish (Figuiredo et al. 2009) demonstrates the potential of *Weissella* sp. to act as a primary pathogen.

Herein, we evaluate the relative acute survival of adult Rainbow Trout from the ARS-Fp-R, ARS-Fp -C, and ARS-Fp -S lines as well as from a commercial source, following an injection challenge with *Weissella* sp. NC36. The effects on fish weight and bacterial load in the fish are examined, and gross and histopathologic changes are described, in order to better understand the pathogenesis of this relatively new disease entity.

METHODS

Bacterial isolate.—*Weissella* sp. strain NC36 was previously isolated and identified by 16S rRNA gene sequencing (Welch and Good 2013) and maintained at −80°C in Man Rogosa and Sharpe (MRS) broth (Difco) supplemented with 25% (v/v) glycerol. Bacteria were cultivated from frozen stock in MRS broth in sealed tubes for 48 h at 30°C.

Experimental fish.—All fish were maintained at the U.S. Department of Agriculture, Agricultural Research Services (USDA-ARS) National Center for Cool and Cold Water Aquaculture (NCCCWA) following NCCCWA Standard Operating Procedures for the Care and Use of Research Animals (Rainbow Trout). The NCCCWA Institutional Animal Care and Use Committee approved this study (protocol 075).

Rainbow Trout, spawned each winter at the NCCCWA, were developmentally synchronized by manipulating water temperature and were hatched within an approximate 2-week period in March. Offspring were certified disease free of common salmonid bacterial and viral pathogens by two independent diagnostic laboratories (Wiens et al. 2013). Individual Rainbow Trout families were challenged with *F. psychrophilum* and selected for differential susceptibility to BCWD (Hadidi et al. 2008; Leeds et al. 2010).

The ARS-Fp-R line in this study was represented by 38 families (with a mean of two fish per family) sampled from a total of 75 families produced and BCWD challenged as part of the NCCCWA breeding program (2011 year-class). The ARS-Fp-S line fish were drawn from 24 families (mean of 3.2 fish per family) sampled from a total of 29 families also produced and BCWD challenged as part of the NCCCWA breeding program. The ARS-Fp-C line fish were randomly sampled from a mixed-family pool consisting of 29 families, but exact family of origin was not known for fish used in this study from this line. All three genetic lines were derived from the same founder population that was developed in 2005 and thus differ only as a result of three generations of selection. Mean survival rates following the BCWD challenge in fish contemporary to those used in this study were 71, 36, and 13% for the ARS-Fp-R, ARS-Fp -C, and ARS-Fp -S lines, respectively. At the time of the BCWD challenge evaluation, the mean body weight was 3.3 g and the fish averaged 80-d posthatch in age. In parallel with this challenge, a subset of the three genetic lines from the 2011 year-class were reared under laboratory conditions and evaluated at 30, 66, and 101 d posthatch (Wiens et al. 2013). In these experiments, as well as in a prior study (Hadidi et al. 2008), we have demonstrated that the relative resistance of the resistant (R) and susceptible (S) lines is a stable trait during fish growth under laboratory rearing conditions.

Experimental design.—Rainbow Trout from the ARS-Fp-R ($n = 75$), ARS-Fp-S ($n = 76$), ARS-Fp-C ($n = 20$) lines and from a pooled population of commercial stock ($n = 24$) from a domestic fish farm were equally divided between two circular, 1,000-L tanks supplied by 12.7°C flow-through water and aeration. Eleven R-line and 10 S-line fish were maintained in a separate 1,000-L tank to serve as a control group. Additionally, ten 55-L tanks (Brute Totes, Rubbermaid) containing one ARS-Fp-R and one ARS-Fp-S fish per tank were simultaneously challenged and used to assess bacterial loads at 3 d postchallenge. Fish averaged 1,094 g (Table 1) and 625 d posthatch in age. The fish had been previously PIT-tagged (Avid Identification Systems,

TABLE 1. Mean (SD) body weight and acute-mortality data summary of Rainbow Trout challenged intraperitoneally with *Weissella* sp. NC36. Percent survival values followed by different lowercase letters are significantly different ($P \leq 0.04$).

Genetic line	*n*	Mean (SD) body weight (g)	Survival (%)	Mean days to death (survivors excluded)
ARS-Fp-R	75	1,049 (356)	44.0 zy	6.6
ARS-Fp-S	76	1,086 (323)	51.3 z	6.6
ARS-Fp-C	20	1,046 (367)	30.0 y	6.2
Commercial stock	24	1,282 (476)	25.0 y	5.9

Norco, California, and Biomark, Boise, Idaho) for identification purposes.

Experimental challenge.—Fish were anesthetized with 90 mg/L tricaine methanesulfonate (Tricaine-S, Western Chemical), weighed, and intraperitoneally challenged with 2.26×10^7 CFU *Weissella* sp., as determined by plate count, in 200 μL phosphate buffered saline (PBS). Intraperitoneal injection of Rainbow Trout with *Weissella* sp. NC36 has demonstrated acute and pronounced disease signs in previous studies (Welch and Good 2013). Control fish were injected with 200 μL PBS. Injections were performed using 1-cm^3 syringes equipped with 26-gauge, 1.27-cm (0.5 in) needles.

Fish were fed daily and monitored for 9 d during the acute phase of disease, and mortalities were removed and recorded daily. Two R-line and two S-line fish demonstrating evidence of disease, including exophthalmia and conjunctival and dermal hemorrhage, were selected for histologic examination. Skin scrapes and gill clips were taken and representative samples of brain, gill, heart, liver, kidney, pancreas, stomach, intestine, and gonad were fixed in 10% neutral buffered formalin for histopathology. Tissues were processed routinely, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E).

On day 3 postchallenge, ARS-Fp-R and ARS-Fp-S fish designated for bacterial load assessment were euthanized via an overdose (>200 mg/L) of Tricaine-S and the spleen and brain were aseptically removed, weighed, and homogenized in PBS (10 μL/mg) using a Bead Beater (BioSpec). Spleen and brain were previously found to harbor relatively high bacterial burdens in naturally infected fish and in experimentally infected Rainbow Trout sampled on day 3, and were thus selected for culture (Welch and Good 2013). Tissue homogenates were serially diluted and 100 μL from each dilution was plated on tryptic soy–blood agar. Colonies were enumerated after incubation for 24 h at 30°C. Sufficient numbers of matched ARS-Fp-C and commercial stock fish were not available and thus load measurements for these lines were not determined.

Statistical analyses.—Binary survival (i.e., died or survived) and time-to-event data (i.e., death or censoring; only survivors at the end of the 9-d challenge were censored) were analyzed using a Cox proportional hazards model in PROC PHREG (SAS version 9.3; SAS Institute, Cary, North Carolina). Because event times were measured with limited precision (i.e., once daily), which resulted in many ties, the EXACT method was used to construct the partial likelihood. Adequacy of the proportional hazards assumption was confirmed using the ASSESS option. The initial full model included fixed effects of genetic line (four levels), challenge tank (two levels), body weight at challenge (linear and quadratic covariate), and all two- and three-way interactions. Effects were sequentially removed from the model if $P > 0.10$.

GraphPad Prism version 5.0 (GraphPad Software, La Jolla, California) was used to graph mortality data and to perform a one-way ANOVA to compare bacterial loads among the genetic lines, and to calculate the Pearson correlation between CFU and body weight. All statistical analyses were run with a significance level of $P < 0.05$. Colony forming unit data were $\log_{10}$ transformed before statistical analysis.

RESULTS

Mortality

Mortality began on day 4 postchallenge and lasted until the termination of the study on day 9 (Figure 1). Genetic line affected survival ($P = 0.05$) and the effect of challenge tank approached significance ($P = 0.08$). Interactions among genetic line, challenge tank, and body weight at challenge did not affect survival ($P \geq 0.15$). In pairwise comparisons, the ARS-Fp-S line had greater survival compared with either the ARS-Fp-C line ($P = 0.04$) or the commercial stock ($P = 0.02$), and the ARS-Fp-R line tended to have greater survival compared with the commercial stock, but it was not significant ($P = 0.09$; Table 1). Estimated risk ratios from the proportional hazards model for the ARS-Fp-S line were 0.52 ± 0.17 (mean ± SD) and 0.49 ± 0.15 compared with the ARS-Fp-C line and commercial stock, respectively, and 0.61 ± 0.18 for the ARS-Fp-R line compared with the commercial stock. No other significant differences were detected among the genetic lines ($P \geq 0.17$).

The linear effect of body weight at challenge was statistically significant ($P = 0.02$); each 10-g increase in body weight increased the hazard of death by 1% (Figure 2). The effect of body weight was also verified independently of the commercial stock, which exhibited the heaviest mean body weight and smallest percent survival, to ensure the population did not bias the results. Interestingly, whereas mortalities from the three ARS genetic lines averaged 164 g heavier than survivors, mortalities in the commercial stock averaged 160 g lighter than survivors. However, the interaction between genetic line and body weight

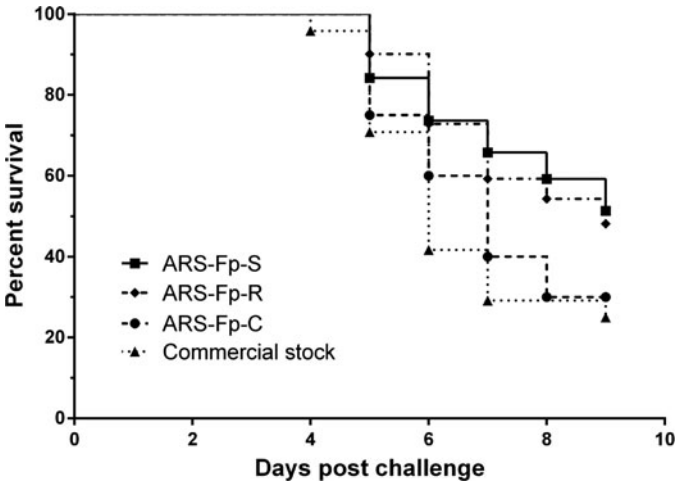

FIGURE 1. Acute survival curves of ARS-Fp-S ($n = 76$), ARS-Fp-R ($n = 75$), ARS-Fp-C ($n = 20$), and a commercial stock ($n = 24$) of Rainbow Trout after intraperitoneal challenge with *Weissella* sp. NC36.

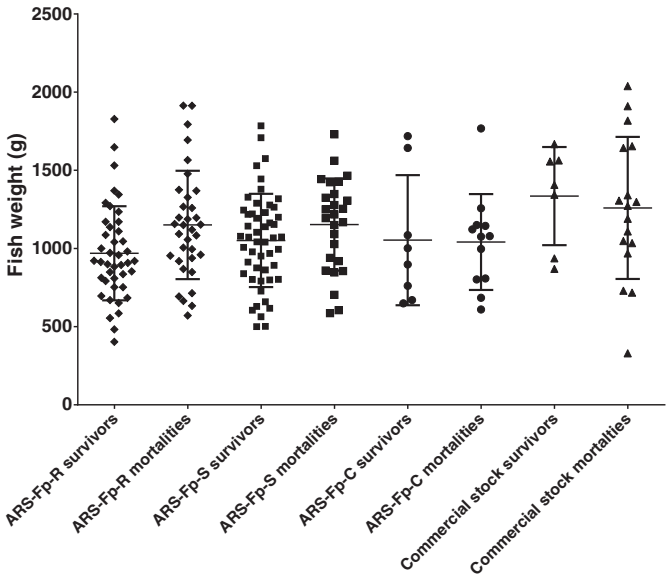

FIGURE 2. Weights on day 0 for survivors and mortalities after intraperitoneal challenge with *Weissella* sp. NC36. A larger mean body weight was observed in mortalities than in surviving Rainbow Trout for the ARS-Fp-R ($n = 75$), ARS-Fp-S ($n = 76$), and ARS-Fp-C ($n = 20$) lines but not for the commercial stock ($n = 24$). The overall linear effect of body weight on mortality was statistically significant ($P = 0.02$). Mid-point horizontal bars represent means and vertical bars represent $\pm$ SD.

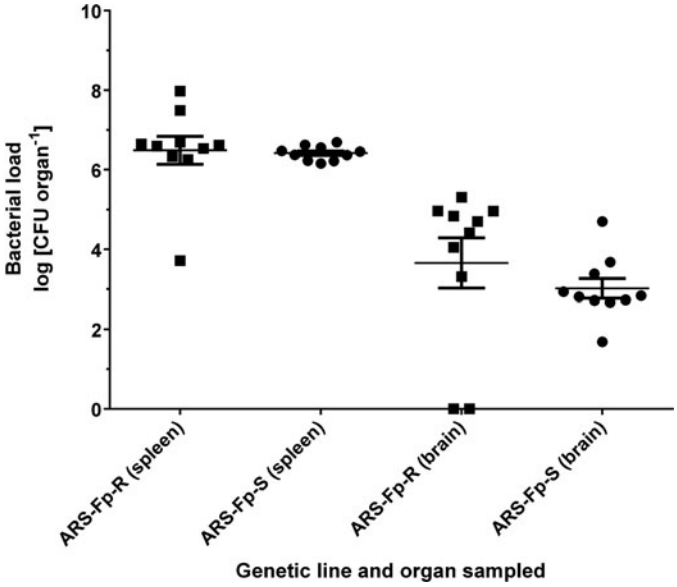

FIGURE 3. Bacterial loads on day 3 in the spleen and brain of ARS-Fp-R ($n = 10$) and ARS-Fp-S ($n = 10$) line Rainbow Trout challenged with *Weissella* sp. NC36, as determined by plate count. Bacterial loads were not statistically different between ARS-Fp-R and ARS-Fp-S fish. A significantly higher bacterial load was present in spleen than in brain for both groups. Mid-point horizontal bars represent means and vertical bars represent $\pm$ SD.

was not statistically significant ($P = 0.15$), presumably because of the fewer number of commercial-stock fish used in the study.

Bacterial Loads

There were no significant differences in mean CFU counts on day 3 between ARS-Fp-R and ARS-Fp-S lines (Figure 3) and no correlation was found between CFU counts and body weight (data not shown). Bacterial loads were significantly higher in spleen than brain within both ARS-Fp-R and ARS-Fp-S fish ($P < 0.0001$; Figure 3).

Clinical Signs and Pathology

Signs of disease in moribund fish included anorexia, lethargy, and recumbent swimming patterns. There was often mild to severe unilateral or bilateral exophthalmia accompanied by variable amounts of scleral and conjunctival hemorrhage (Figure 4a). Fish exhibited mild to moderate hemorrhage and congestion within the visceral adipose tissue and small areas of redness surrounded the injection site. There was occasional ascites within the pericardial and coelomic cavities that was accompanied by renomegaly and splenomegaly. Infrequently, multifocal to locally extensive meningeal and cerebral hemorrhage was present on the olfactory lobes and optic tectum of the brain (Figure 4b). No significant changes were noted in gill clips or skin scrapes.

On histologic examination of four fish, the retrobulbar space was infiltrated and expanded by low to marked numbers of neutrophils, lymphocytes, and epitheloid macrophages. Inflammatory cells disrupted the tunica media and tunica adventitia of small arteries, which was accompanied by extensive vascular congestion, hemorrhage, and proteinacious fluid (Figure 4c). Although infiltration into the optic nerve was not observed, inflammatory cells often lined the outer nerve sheath (Figure 4c). Similar inflammatory infiltrates expanded the epicardium of the heart with infiltration into the myocardial interstitium, where there was scattered myofiber degeneration and necrosis (Figure 4d). In one fish, epicardial inflammation was associated with scores of coccobacilli with frequent phagocytosis by histiocytic cells (Figure 4d). Sections of brain, gill, liver, spleen, cranial and caudal kidney, gastrointestinal tract, and gonad exhibited no significant histologic findings.

DISCUSSION

This study examined whether BCWD resistant, BCWD susceptible, and control Rainbow Trout lines displayed differential susceptibility to an unrelated pathogen, *Weissella* sp. NC36. The absence of a significant survival difference between the ARS-Fp-R and ARS-Fp-S lines indicates that during the acute phase of disease, the mechanisms responsible for BCWD resistance are not effective in 1,000-g fish challenged with *Weissella* sp. NC36. The lack of demonstrated survival differences between these two genetic lines is also consistent with a preliminary infection trial that used 500-g fish and concluded after 92 d (results not shown). As further confirmation, examination of *Weissella* loads in both spleen and brain showed no difference between

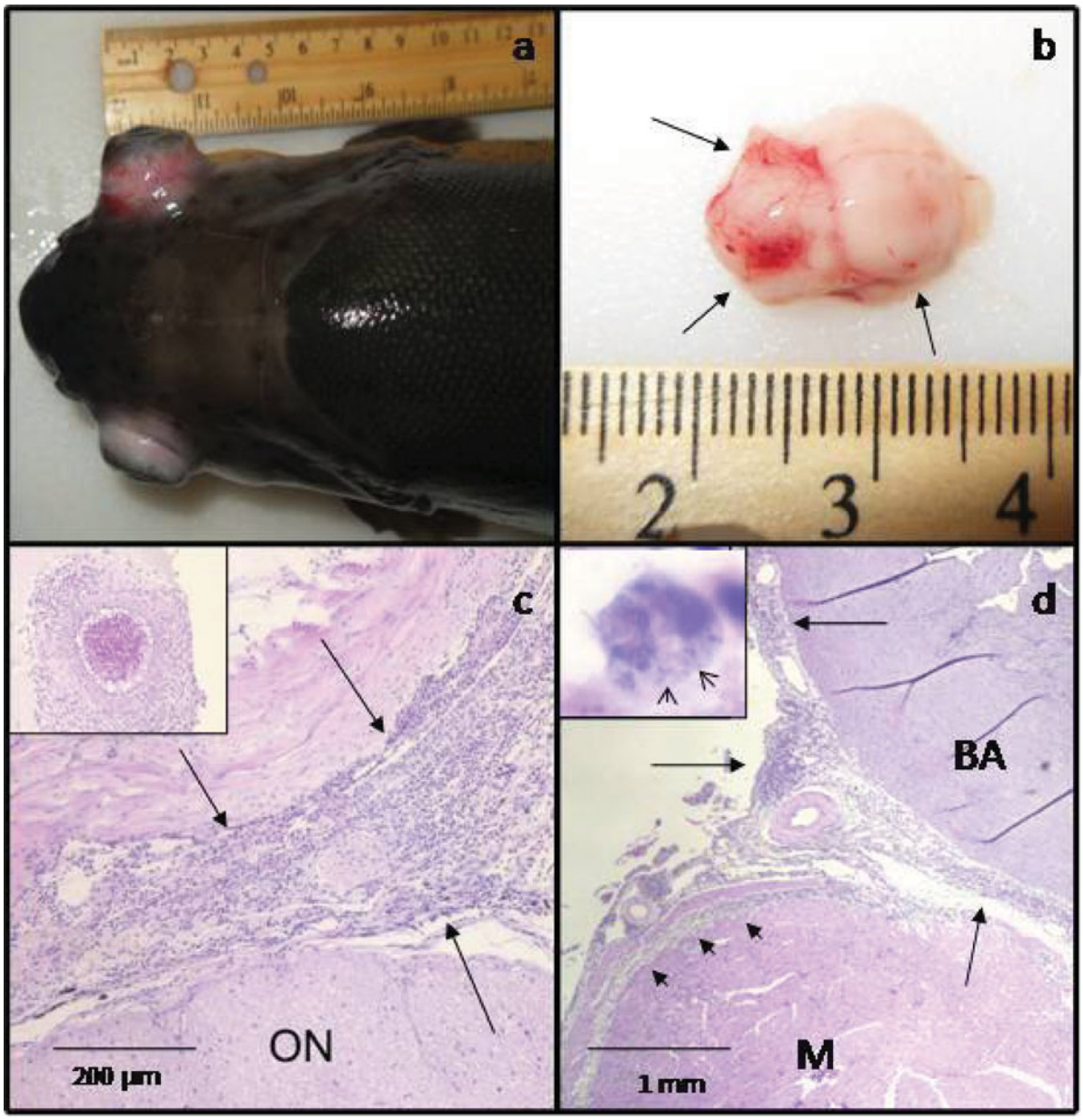

FIGURE 4. Gross and microscopic necropsy findings in Rainbow Trout challenged with *Weissella* sp. NC36. (**a**) Bilateral exophthalmia with conjunctival and scleral hemorrhage and congestion. (**b**) Cerebral hemorrhage and congestion along the olfactory lobes and optic tectum (arrows). (**c**) A mixed inflammatory infiltrate of neutrophils, lymphocytes, and macrophages expands the retrobulbar space (arrows). Inflammation borders the optic nerve (ON) but does not infiltrate neural tissue. Insert: granulomatous vasculitis of a periorbital artery. (**d**) Mixed inflammatory cells (long arrows) expand the epicardium along the bulbous arteriosus (BA) and infiltrate (small arrows) into the superficial myocardium (M). Insert: intrahistiocytic bacteria (arrowheads) present within areas of epicardial inflammation. [Figure available online in color.]

the ARS-Fp-R and ARS-Fp-S lines. We previously reported that resistant-line fish, at a similar size to fish in this study (800 g), challenged with *F. psychrophilum*, had a 14-fold lower bacterial load in the spleen compared with BCWD-susceptible-line fish (Hadidi et al. 2008). This finding suggests that differential survival among lines to *F. psychrophilum*, and a lack of survival difference to *Weissella* sp. NC36, is related to *F. psychrophilum*-specific mechanisms controlling bacterial clearance or trafficking.

These data have practical relevance as they suggest ARS-Fp-R line fish grown at *Weissella*-endemic farms are not at greater risk due to an unfavorable correlated response to selective breeding for BCWD resistance. In addition, the ARS-Fp-R line does not appear to be at increased risk compared with a commercial line of Rainbow Trout. However, it is important to note that a relatively small number of commercial-line fish were used in this experiment, and the small sample size may present a biased representation of the commercial population. The relatively poor

survival of the ARS-Fp-C line was unexpected, but this result should likewise be interpreted with caution because of the small sample size and limited knowledge of genetic diversity represented in this study. We are not aware of any published reports on the heritable nature of *Weissella* resistance, nor are the family sizes ($n \approx 3$ fish per family) of the ARS-Fp-R and ARS-Fp-S lines used in this study large enough to estimate between-family variation.

The lack of difference between the ARS-Fp-R and ARS-Fp-S lines in response to a *Weisella* challenge or an infectious hematopoietic necrosis virus (IHNV) challenge (Wiens et al. 2013) suggests that breeding for BCWD resistance does not produce detectable positive or negative influence on susceptibility to distantly related gram-positive or viral pathogens. Similarly, no correlation between BCWD and IHNV resistance were observed in an unrelated population of Rainbow Trout (Overturf et al. 2010). A study by Gjøen et al. (1997) in Atlantic Salmon *Salmo salar* describes positive correlation for resistance to *Aeromonas salmonicida*, *Vibrio salmonicida*, and *V. anguillarum*. Henryon et al. (2005) demonstrated a weak negative genetic correlation for resistance to *F. psychrophilum* and *Yersinia ruckeri* in Rainbow Trout. In summary, there are limited data to support either a strong adverse or a strong favorable correlation between resistances for different diseases (Ødegård et al. 2011). Current efforts are underway to examine whether the ARS lines of trout differ in resistance to gram-negative pathogens or other strain variants of *F. psychrophilum*.

This study has provided new information on the pathophysiology of Weissellosis. Intraperitoneal challenge with *Weissella* sp. NC36 produced mortality and disease signs consistent with those described for natural infection (Liu et al. 2009; Figueiredo et al. 2012; Welch and Good 2013). At 12.7°C, water temperature in this challenge study was substantially colder than the seasonal 18–20°C water described during the North Carolina farm outbreak (Welch and Good 2013). This implies that given a portal of entry and sufficient dose, *Weissella* sp. NC36 has the ability to cause significant morbidity and mortality at a wide range of temperatures relevant to salmonid aquaculture.

Clinical signs and gross lesions were associated with microscopic findings in the four fish necropsied. Exophthalmia and ocular hemorrhage appeared to have occurred secondary to retrobulbar granulomatous inflammation and vasculitis, probably from septicemia. Although cerebral hemorrhage was present, microscopic changes were not apparent in the brain of the limited number of fish examined. Gross cerebral hemorrhage and consistent recovery of bacteria from brain swabs in this study and the natural outbreak in North Carolina (Welch and Good 2013) suggest this organ is a target for *Weissella* sp. and a larger sample size may help deduce pathogenesis of this lesion. Another consistent finding was granulomatous myocarditis associated with intracellular bacteria and scattered myofiber necrosis. Damage to this vital organ may play a role in mortality and should be a target for future bacteriology and pathogenesis studies.

It is unlikely that the marginal significance of tank effect was due to variation in environment or husbandry. Both tanks were directly fed by the same water source with identical water flow, temperature, and water quality parameters. Similarly, they were under the same light : dark regime and feeding conditions.

The linear effect of body weight at challenge was significant, and higher mortality occurred in larger-sized fish. This corresponded to case reports that described a greater incidence of Weissellosis in larger, market-weight fish during farm outbreaks (Welch and Good 2013). Pathophysiologic and environmental factors that influence the association between disease and fish size or age are unknown at this time, but remain an area of interest. As splenic and cerebral CFU counts did not significantly correlate with body weight, this suggests that morbidity and mortality are not directly related to the relative ability of smaller fish to clear or inhibit bacteria.

A sampling period through the chronic stage of disease may provide a more complete insight into the kinetics of infection and innate disease resistance mechanisms involved in Weissellosis. Similarly, further studies are needed to explain disease factors associated with fish size or age. Emergence of *Weissella* sp. in market-size Rainbow Trout on three continents, the demonstration of disease over a wide temperature range, and the relatively low survival rate of the pooled commercial fish population indicate that Weissellosis will remain significant for the salmonid aquaculture industry.

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